

A Reference RNA for QPCR Assay Standardization

Metrology and Standards Needs for
Gene Expression Technologies:
Universal RNA Standards
March 28-29, 2003

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Outline:

- Introduction
- Controls, references and standards in QPCR
- Uses for reference RNA
- The importance of standard curves
- Confidence intervals and data comparison
- Discussion

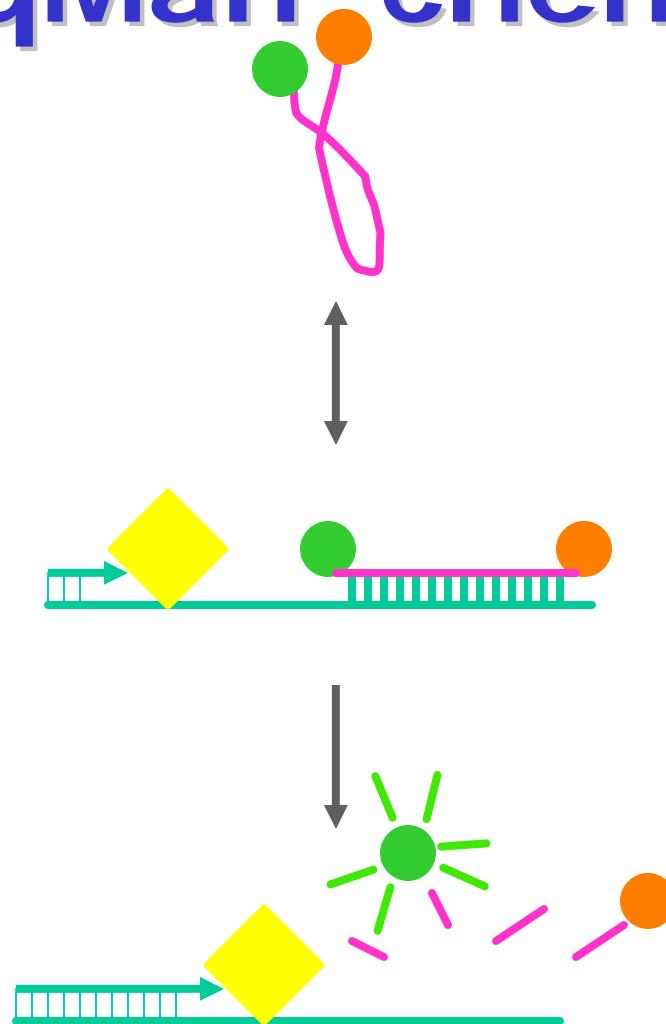
PCR Amplification

PCR: Correlation of amount of amplified DNA to amount of initial target DNA

$$Y = X (1 + E)^n$$

- Y = PCR amplified quantity
- X = target DNA quantity
prior to PCR
- E = amplification efficiency
- n = number of cycles

TaqMan[®] chemistry

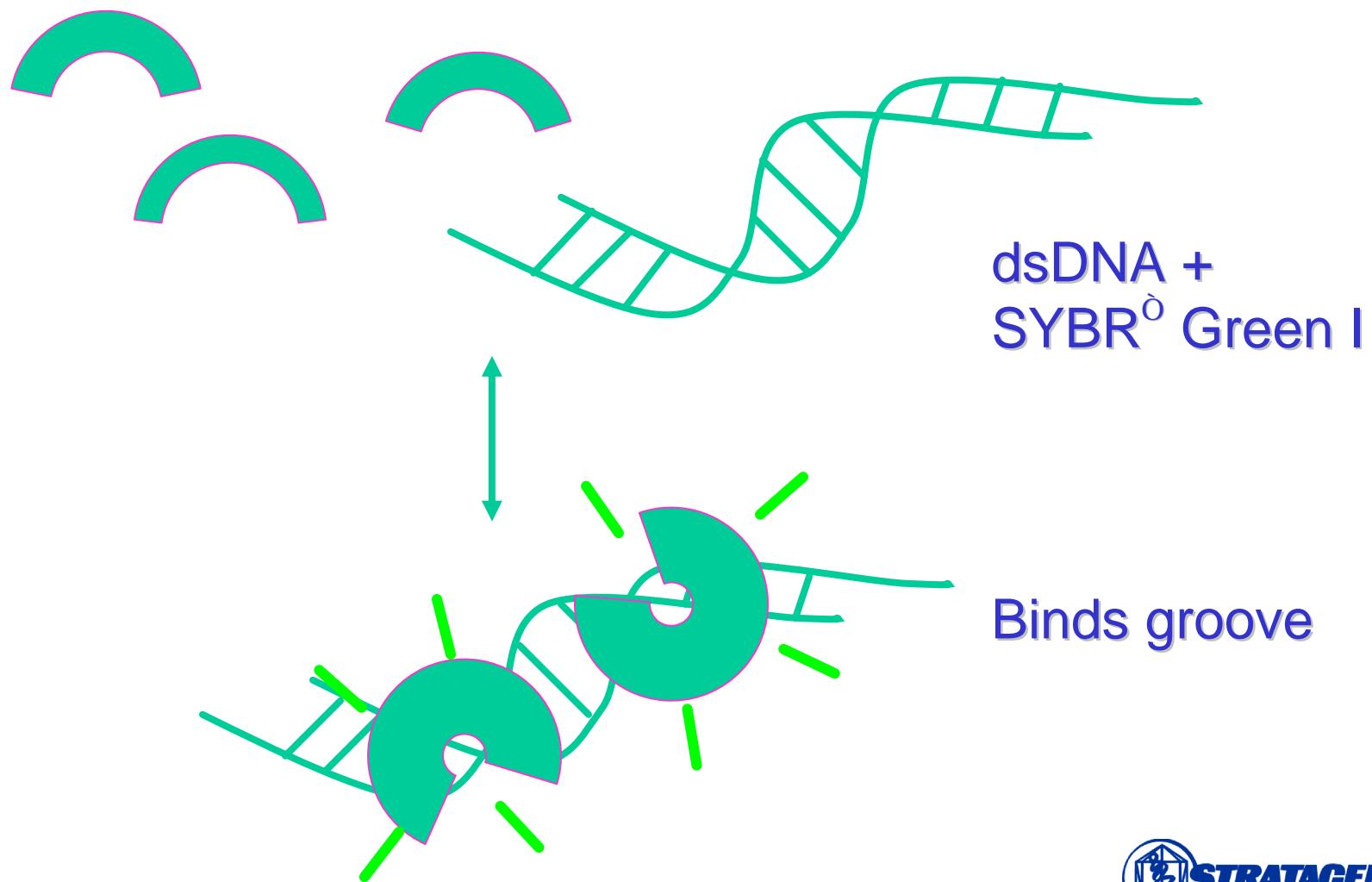


Probe

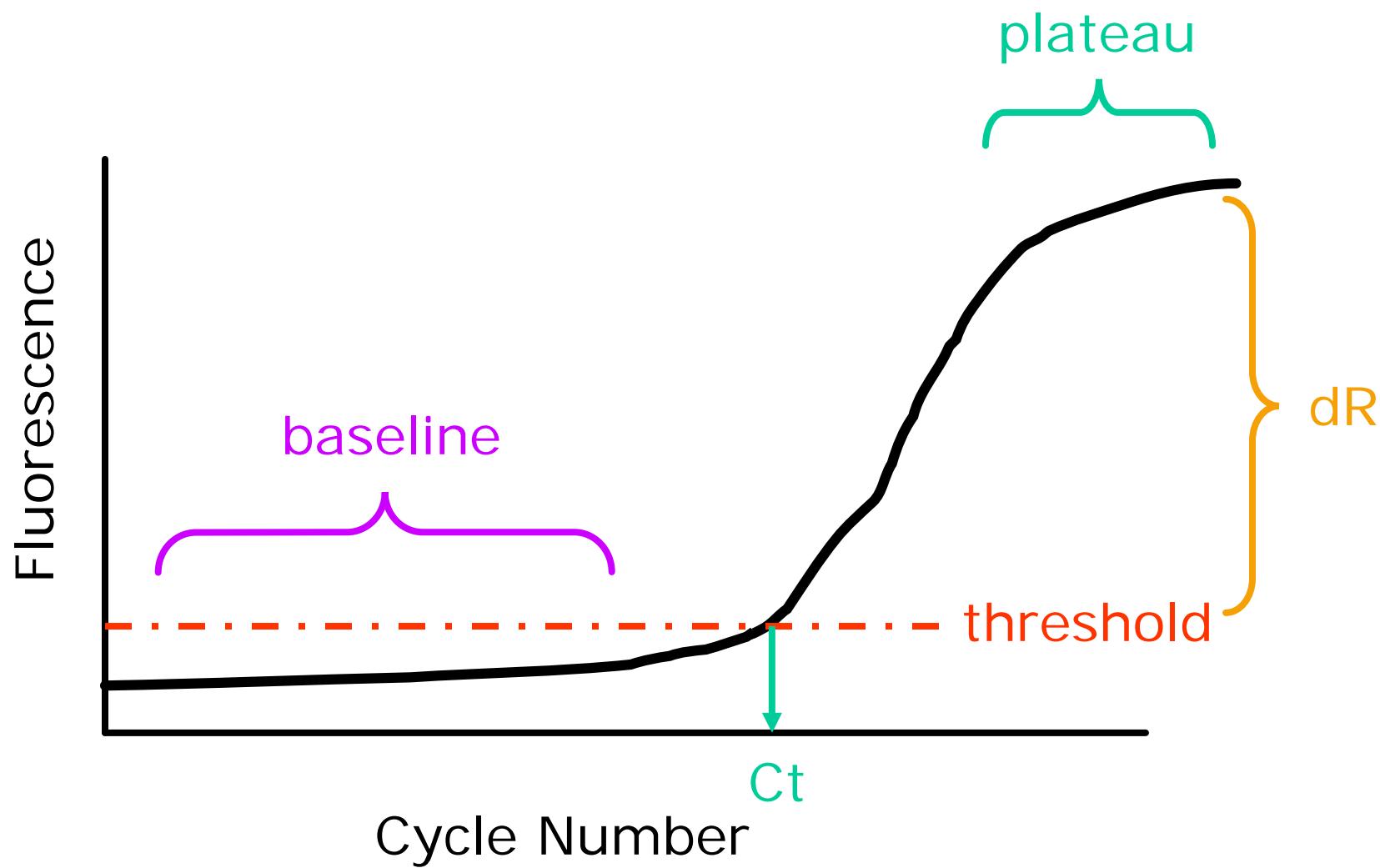
Target and
enzyme

Hydrolysis

SYBR[®] Green I Chemistry

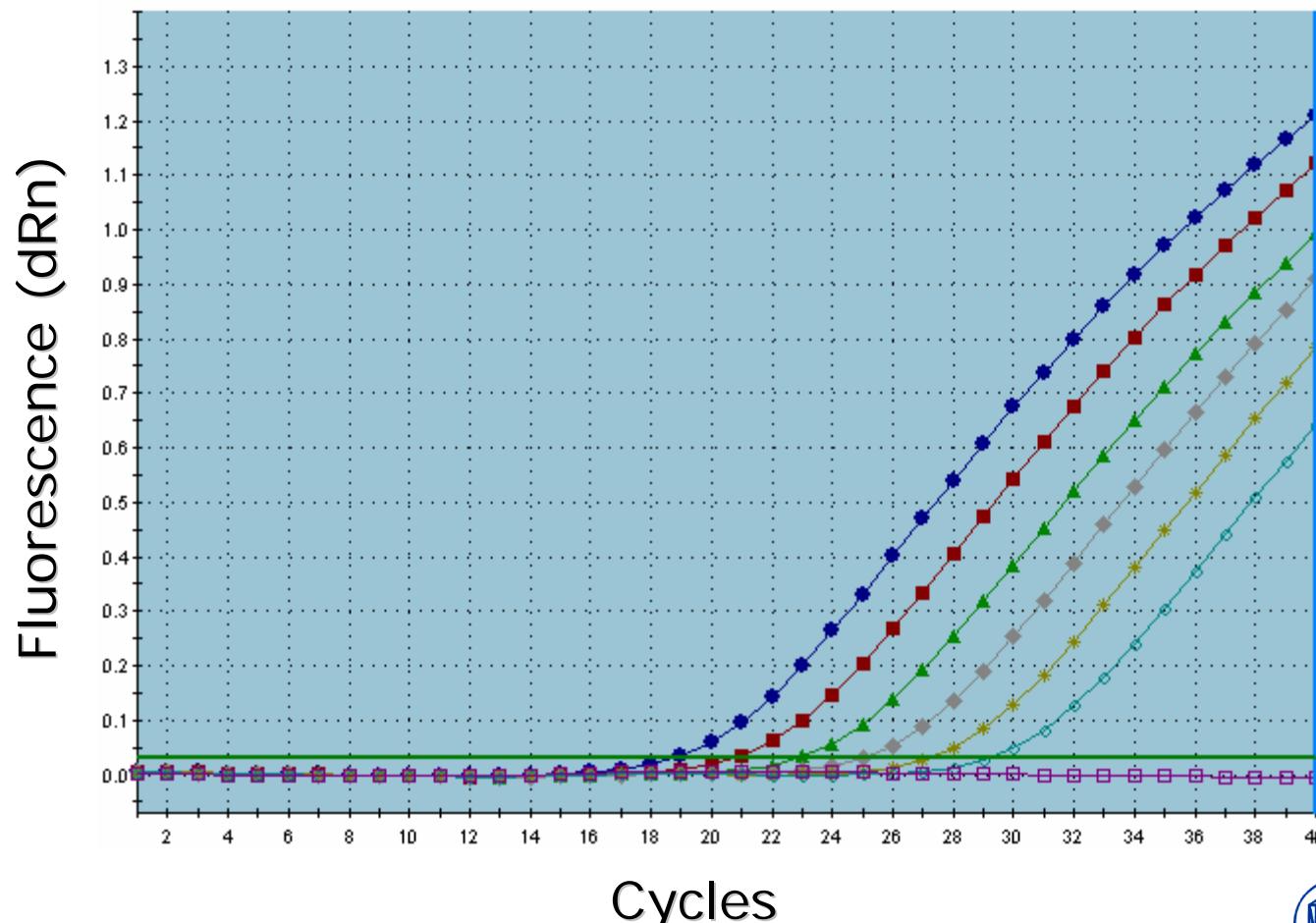


Amplification Plot Terms



Amplification Plot

GUS - 4x Dilution, starting at 1000 ng (lin/lin)



Variability in Determining Initial Target Concentration

...can be introduced by:

Instrumentation
Reagents
Template
Operator
Analysis

The Template in a Universal Reference for QPCR can be...

- Total RNA
- Poly(A)_n-containing RNA
- Synthetic RNA
- cDNA

Quantitative PCR Human Reference Total RNA

- Good gene representation.
- Precise determination of RNA concentration.
- Extensive quality control.
- Production in large batches.
- Convenient buffer.

Cell lines Used in the Production of QPCR Human Reference Total RNA

Glioblastoma, brain

Adenocarcinoma, cervix

Adenocarcinoma, mammary gland

Embryonal carcinoma, testis

Liposarcoma

Melanoma, skin

Hepatoblastoma, liver

Histiocytic lymphoma, macrophage/histiocyte

Plasmacytoma/myeloma, B lymphocyte

Lymphoblastic leukemia, T lymphoblast



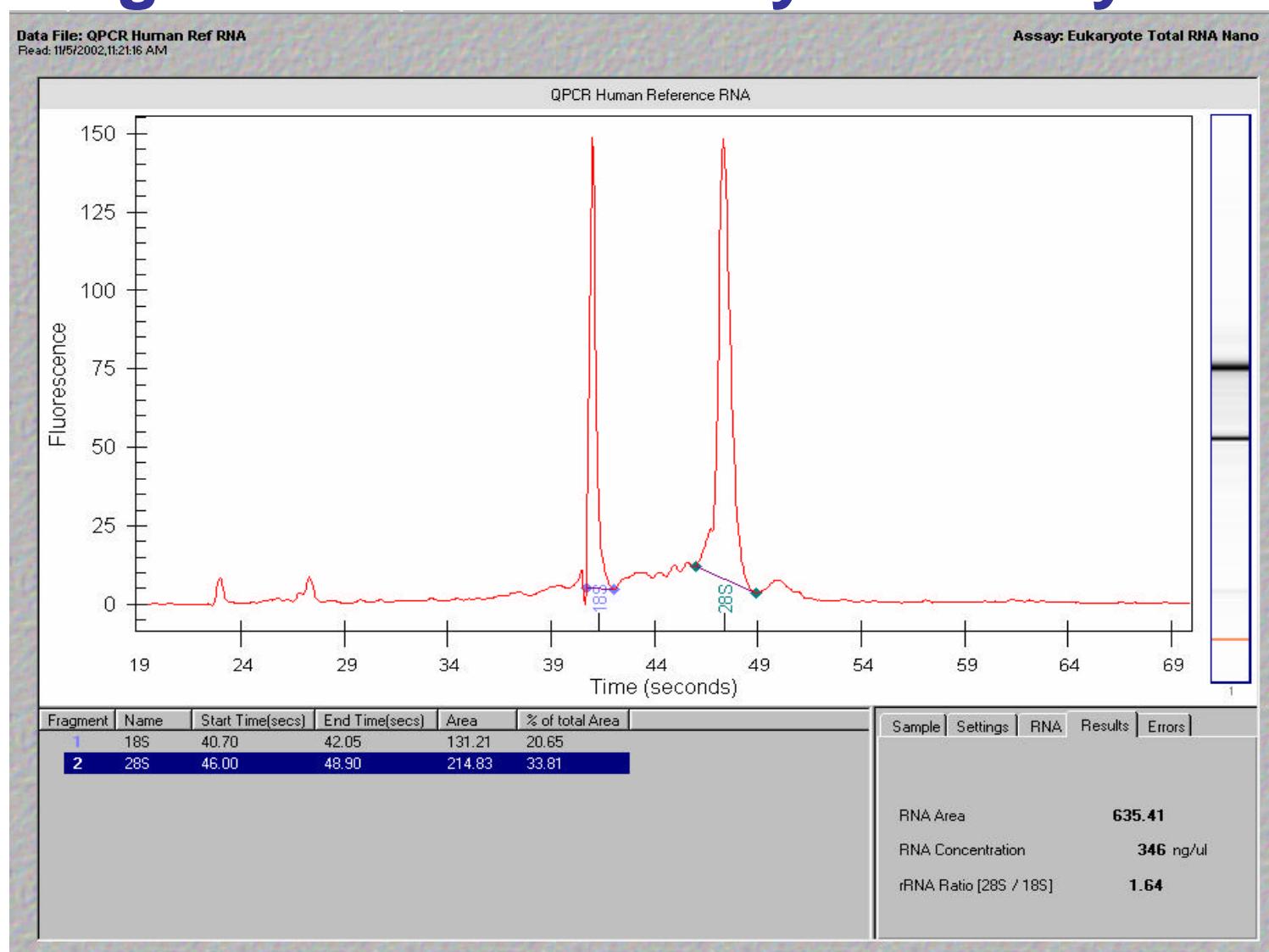
Determination of RNA Concentration

- **A260/A280**
- Olive Green
- Agilent Bioanalyzer
- **Ribo-Green**

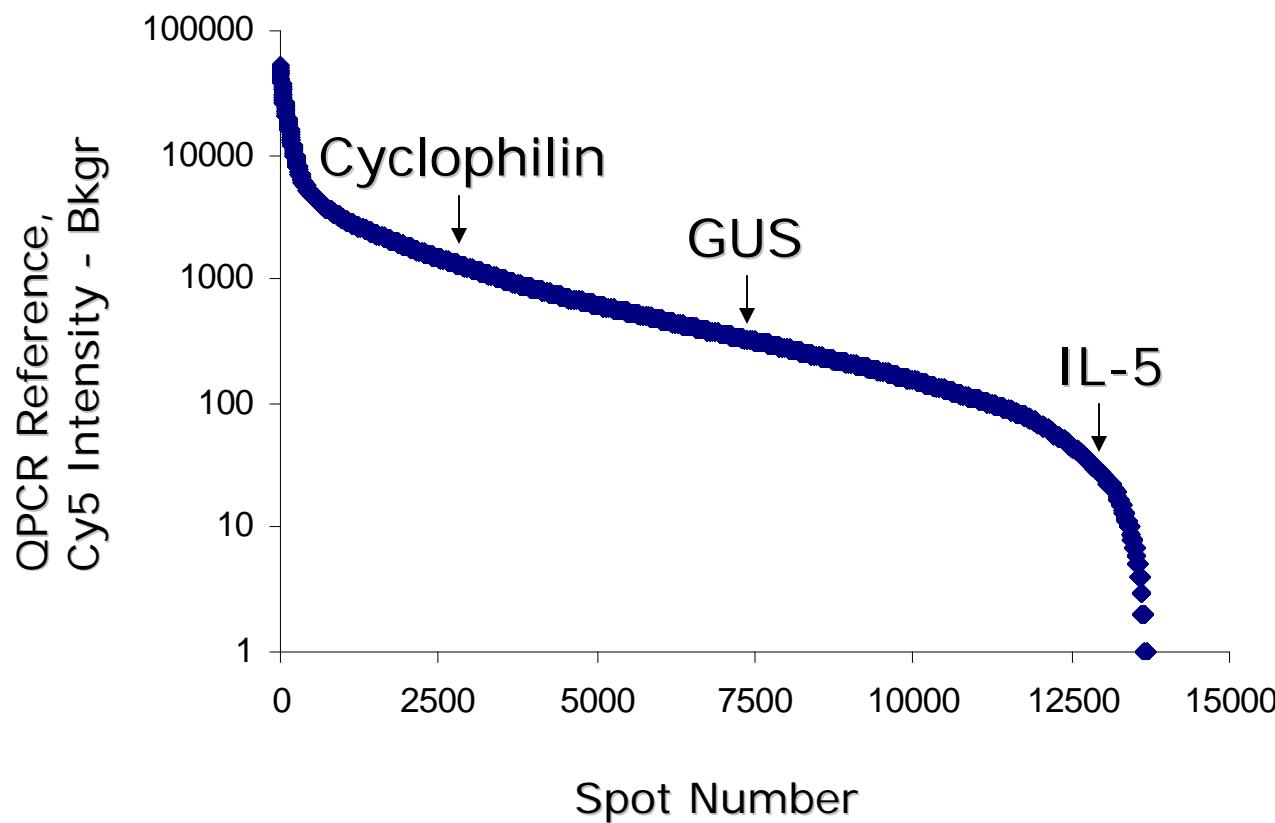
Quality Control for Quantitative PCR Human Reference Total RNA

- 1. Spectrophotometry**
 $OD_{260/280} > 1.8$
- 2. Formaldehyde-agarose gel electrophoresis**
18S and 28S ribosomal bands are without degradation
- 3. Agilent 2100 Bioanalyzer**
18S and 28S ribosomal bands are without degradation,
rRNA Ratio [28S/18S] > 1.5
- 4. RNA concentration** is determined using RiboGreen Dye and
by spectrophotometry
- 5. RNase contamination testing** (2hr incubation at 37° C)
- 6. Evaluation of gene representation using microarrays**
- 7. DNA-contamination (QPCR)**
- 8. Target quantification** using TaqMan[®] probes and SYBR[®] Green I

Agilent 2100 Bioanalyzer Analysis



QPCR Reference RNA: High, Medium, Low Abundance Targets Identified on a 12,000-Spot Agilent Human cDNA Microarray



High, Medium and Low Abundant Targets Analyzed with TaqMan[®] Probes (PDARs)

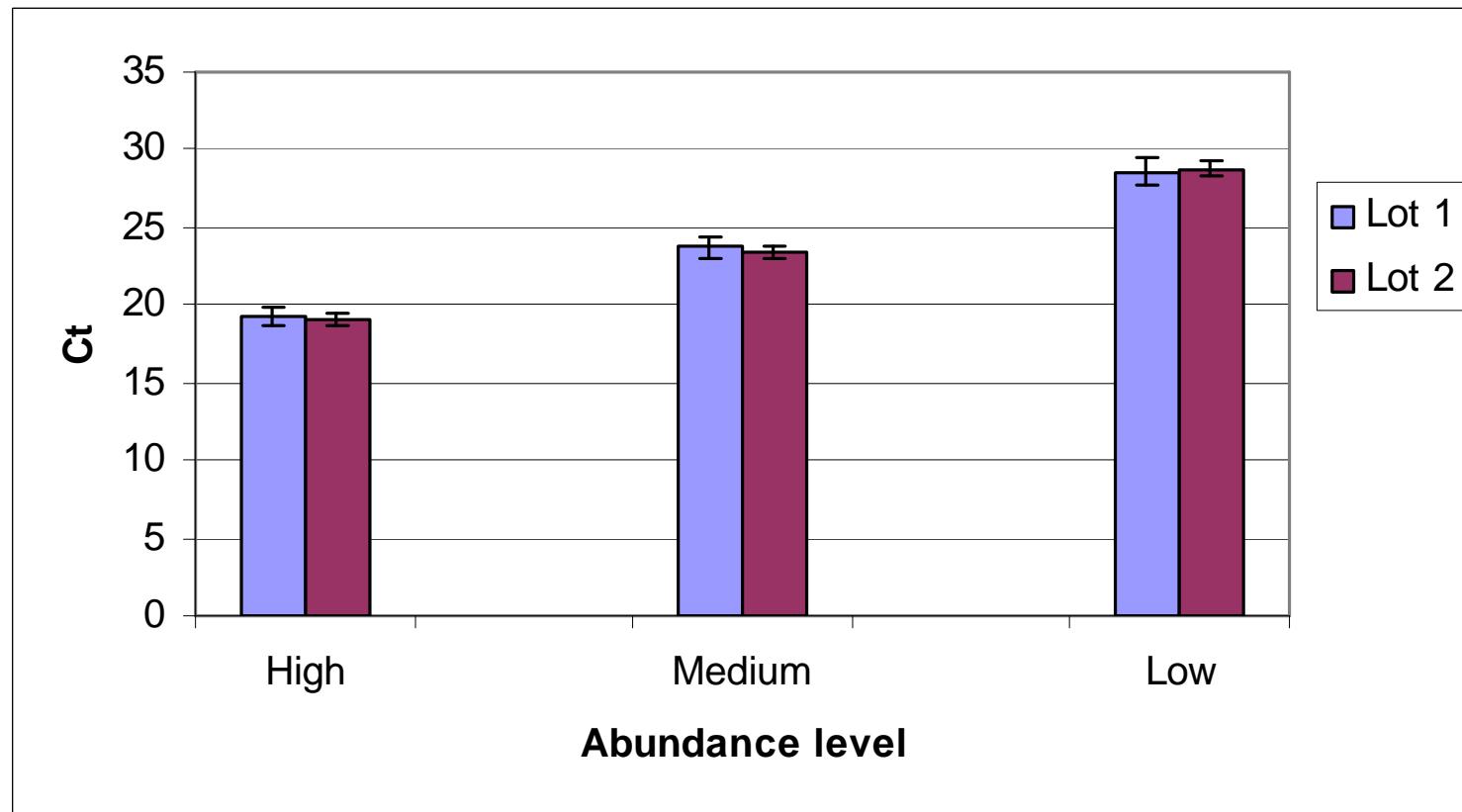
| b2-Microglobulin | | |
|-------------------------|-------|-------|
| | Lot 1 | Lot 2 |
| 100ng | 19.3 | 19.1 |
| 10ng | 22.2 | 22.0 |
| 1ng | 25.9 | 25.3 |
| 0.1ng | 29.2 | 29.0 |
| NTC | no ct | no ct |

| TBP | | |
|------------|-------|-------|
| | Lot 1 | Lot 2 |
| 100ng | 23.7 | 23.4 |
| 10ng | 26.7 | 26.5 |
| 1ng | 30.2 | 29.5 |
| 0.1ng | 34.5 | 34.5 |
| NTC | no ct | no ct |

| GUS | | |
|------------|-------|-------|
| | Lot 1 | Lot 2 |
| 100ng | 24.6 | 24.3 |
| 10ng | 26.9 | 26.5 |
| 1ng | 30.5 | 29.8 |
| 0.1ng | 34.6 | 33.4 |
| NTC | no ct | no ct |

| IL-5 | | |
|-------------|-------|-------|
| | Lot 1 | Lot 2 |
| 100ng | 28.6 | 28.8 |
| 10ng | 32.2 | 32.8 |
| NTC | no ct | no ct |

Lot-to-Lot Variation in QPCR Reference RNA



β 2-Microglobulin (high)
TATA Box Binding Protein (medium)
IL-5 (low)

Universal RNA Standards Workshop, March 28-29, 2003



In QPCR there is a need today...

- for a RNA laboratory standard used for instrument and assay validation
(David Ginzinger at UCSF, Rupinder Grewal at Millennium, ABI for "assay-on-demand")
- for a worldwide standard to compare results across countries and platforms
(European Consortium of Clinical Laboratories)

Standard Curve Quantitation Versus Comparative Quantitation

Standard Curve Quantitation:
Gene of interest (GOI)
Normalizer
Passive reference dye

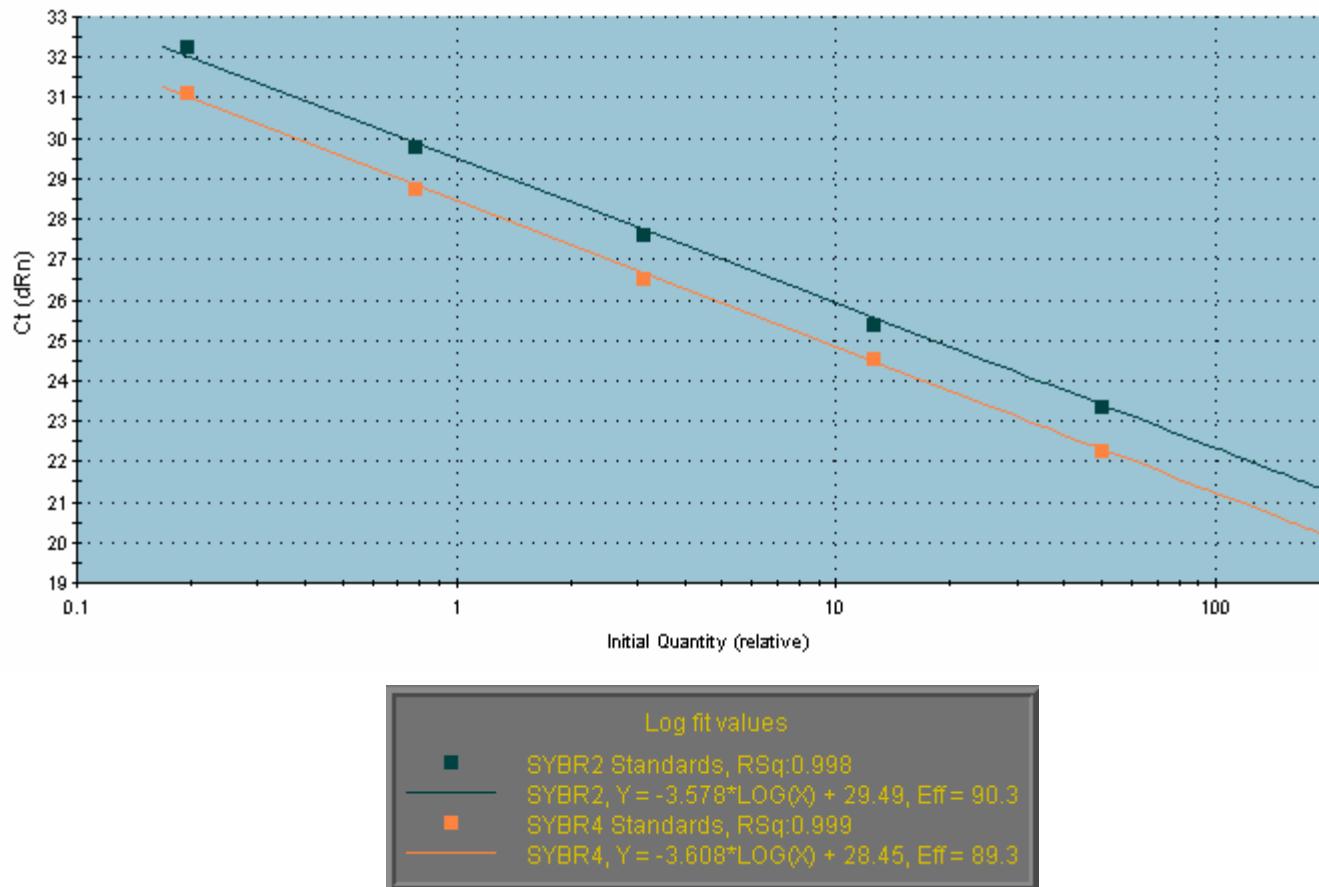
Relative Comparison:
Gene of interest
Normalizer
Calibrator
Passive reference dye

Common Terms Used for Controls in QRT-PCR and QPCR

- Control
- Reference
- Normalizer
- Standard
- Calibrator

Assay Validation

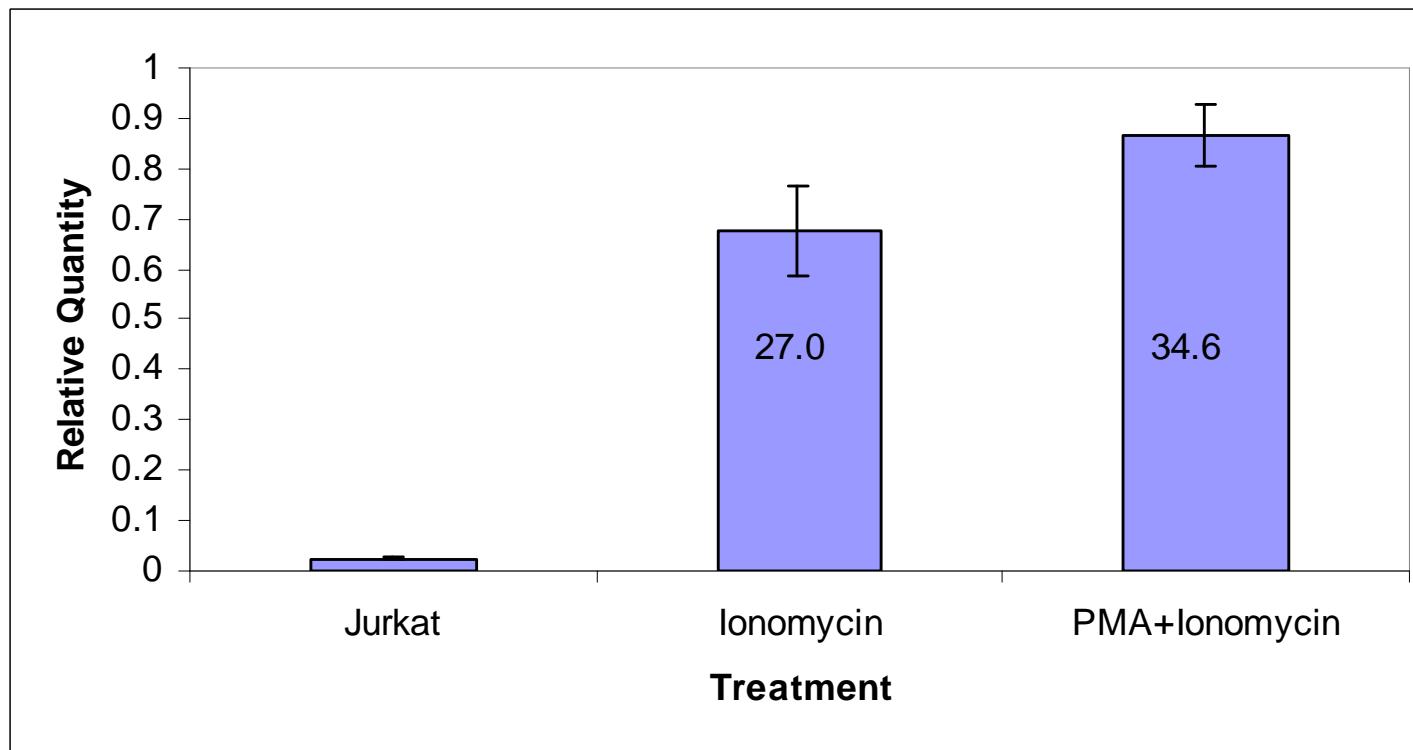
Assay Validation



A SYBR Green I assay for PMCA was validated using QPCR reference RNA. The reference gene was β 2-microglobulin.

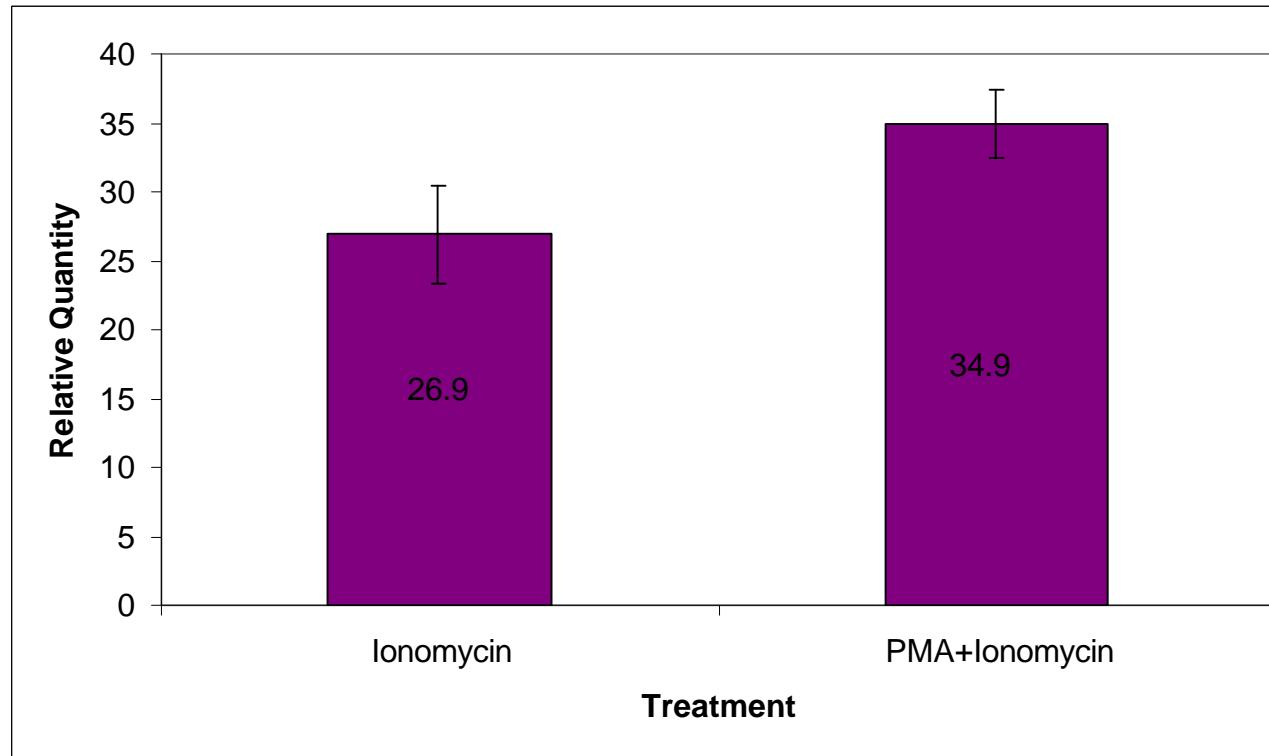
Assay Development

Relative Quantification with QPCR Reference RNA



Target is PMCA 1; SYBR Green I detection;
Normalizer is β 2-microglobulin;
Calibrator is QPCR Reference Total RNA;
Total RNA from untreated Jurkat cells, treated with
Ionomycin, or treated with Ionomycin/PMA.

Relative Quantification of PMCA 1 mRNA



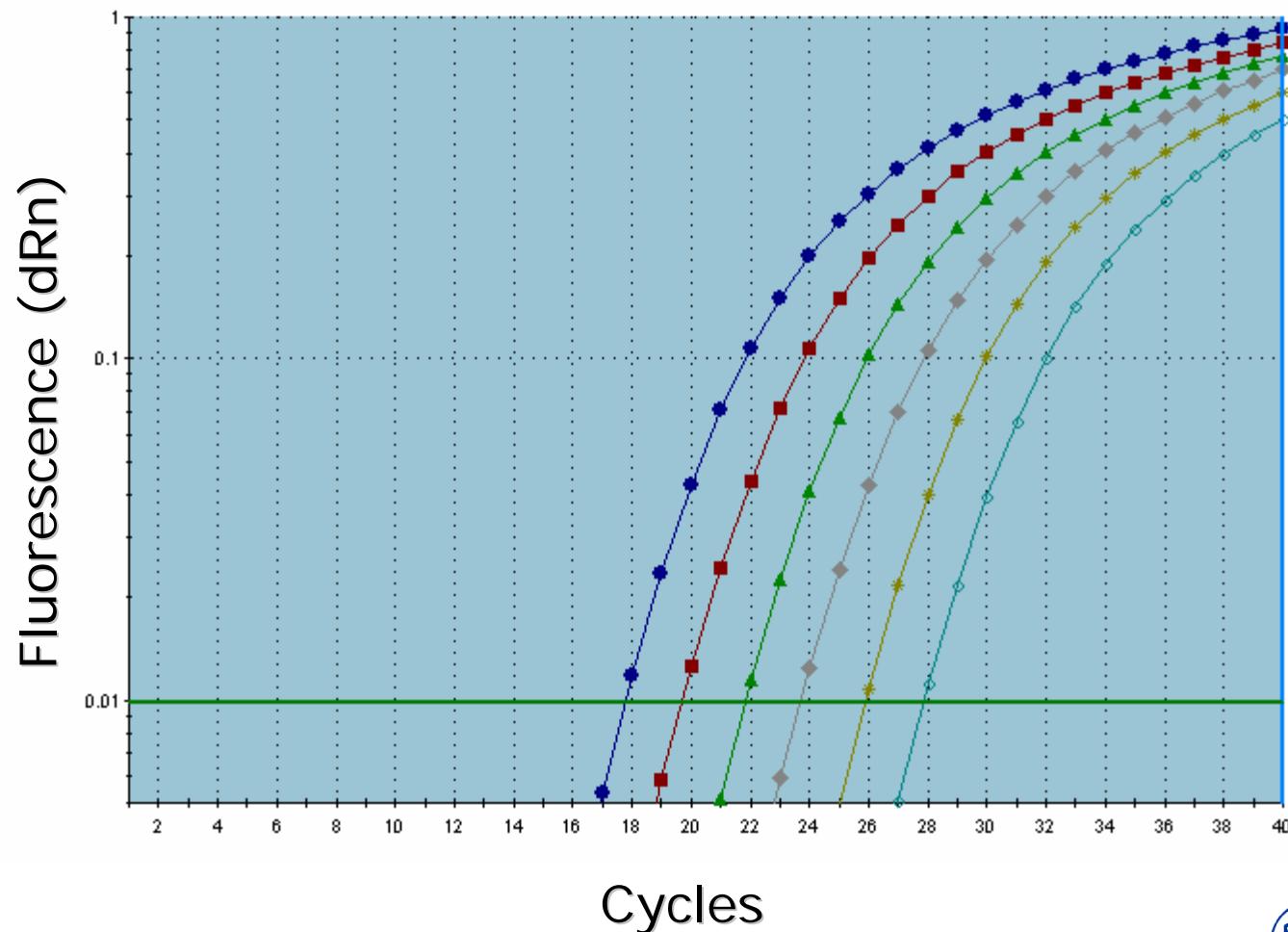
Target is PMCA 1; SYBR Green I detection;
Normalizer is β 2-microglobulin;
Calibrator is total RNA from untreated Jurkat cells;
Experimental RNAs are from Jurkat cells treated with
Ionomycin, or treated with Ionomycin/PMA.



Assay Standardization

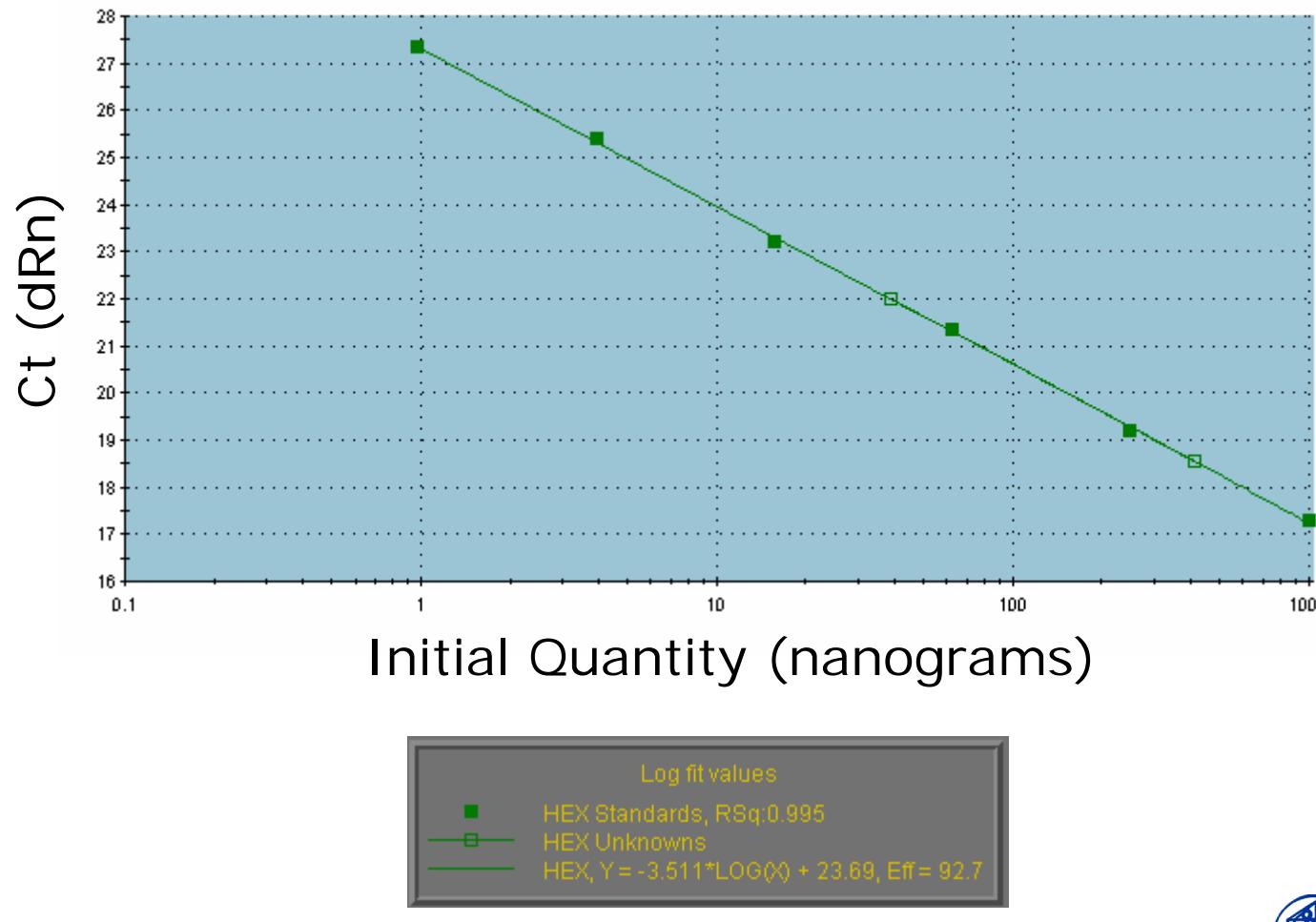
Amplification Plot

GUS - 4x Dilution, starting at 1000 ng (lin/log)



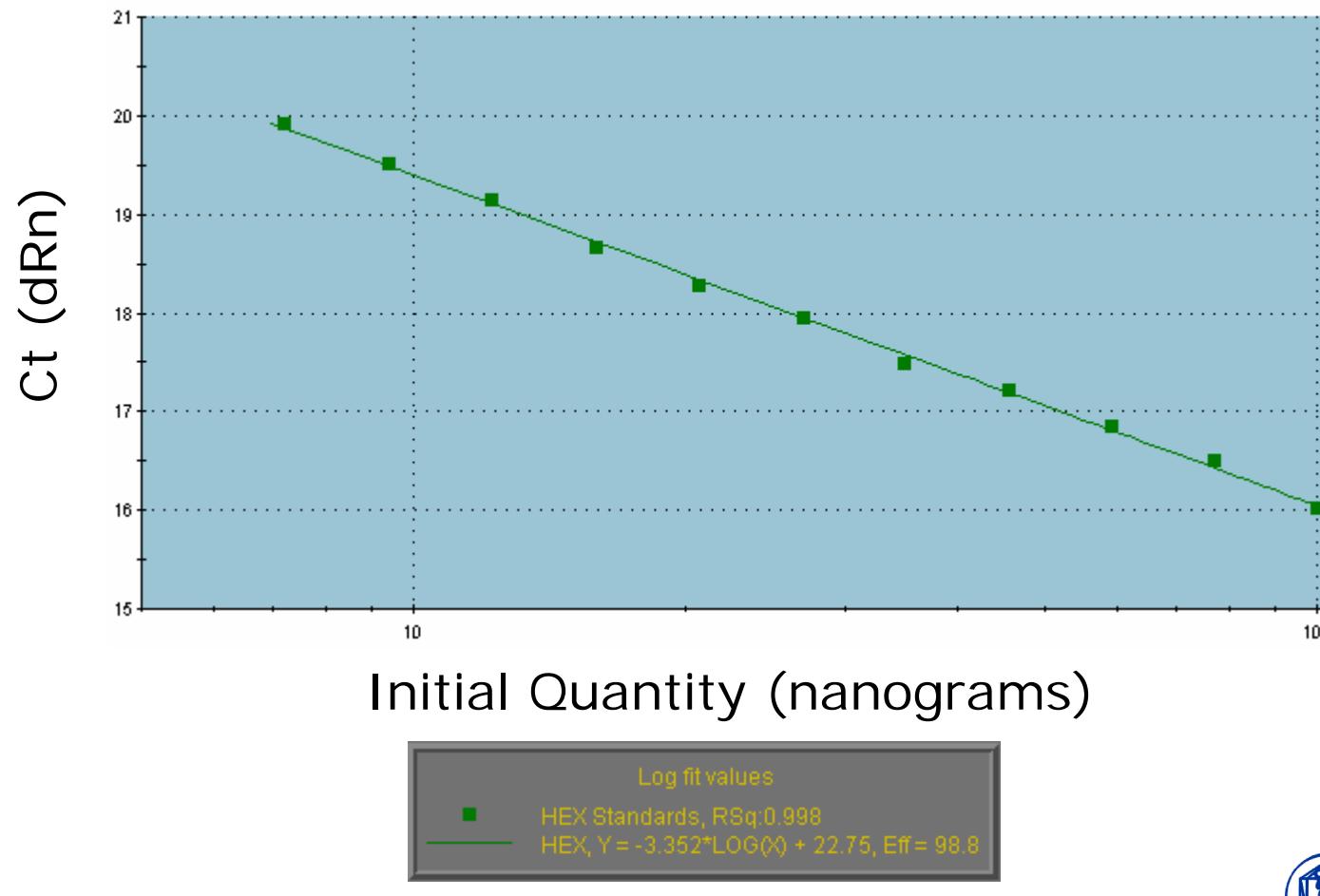
Standard Curve

GUS - 4x Dilution, starting with 1000 ng



Standard Curve

Cyclophilin - 1.3x Dilution, starting with 100 ng



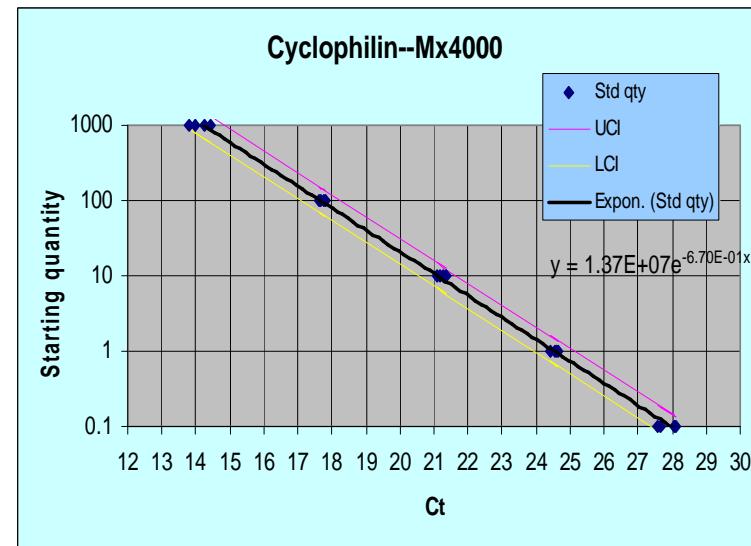
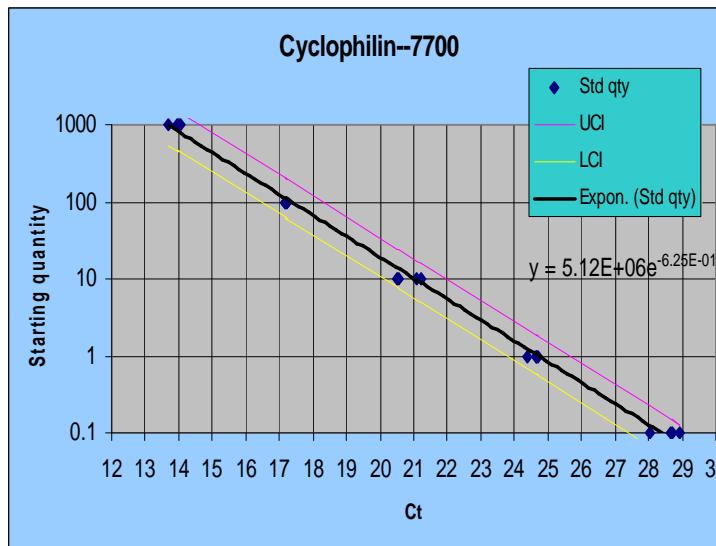
Describing a Standard Curve

1. Linear Range
2. Standard deviation of replicates and R²-value
3. Confidence interval
4. Slope of the best-fit line
5. Y-axis intercept

Mathematical Model for Comparison of Two QRT-PCR Runs

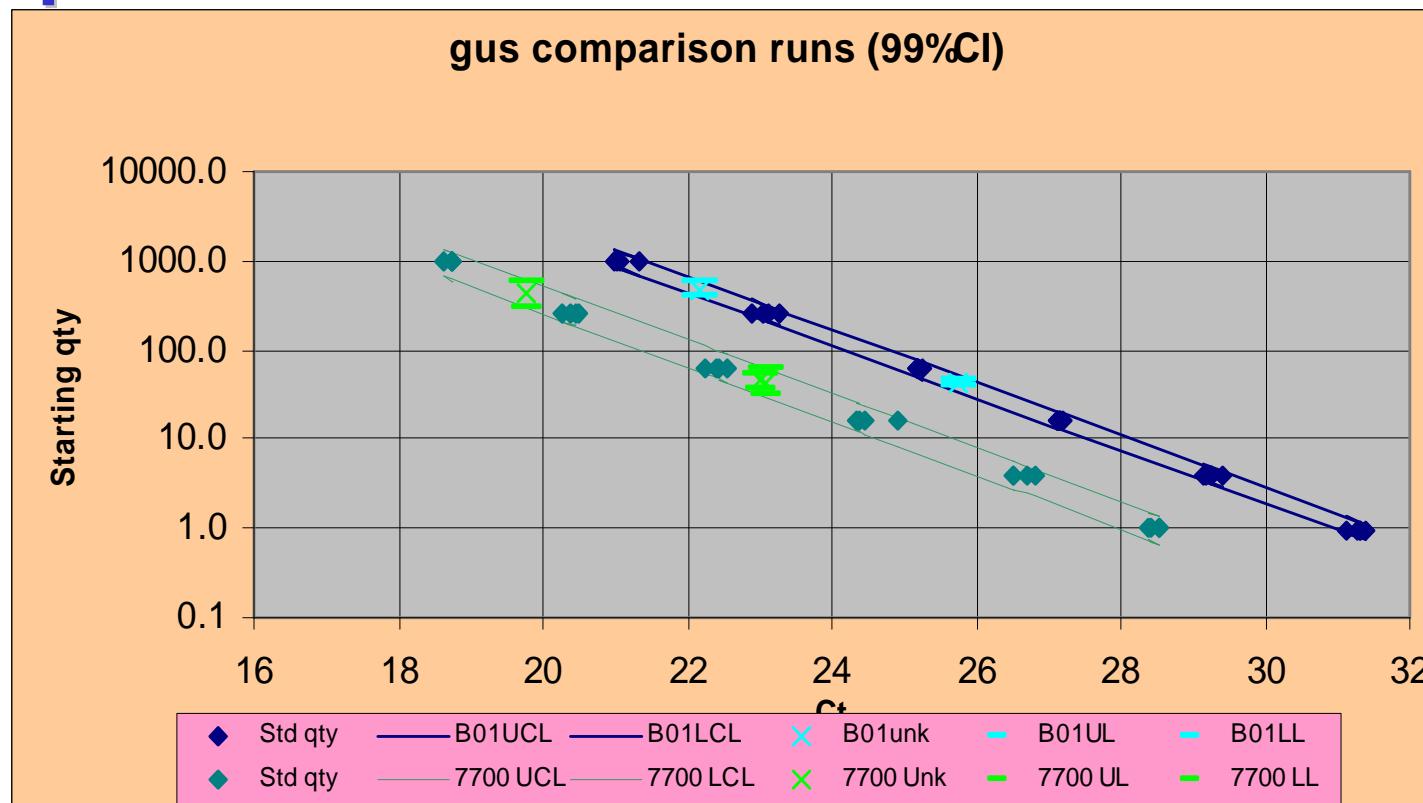
- Decide on number of replicates.
- Generate a standard curve with QPCR Reference Total RNA.
- Calculate Confidence Intervals (CI).
- Compare quantities from runs on different days or on different platforms.
- Apply modified t-test.
- Decide if the two “Unknown” quantities were different.

Intra-Laboratory Comparisons



Target is cyclophilin; TaqMan[®] probe detection;
Template is QPCR Reference RNA.

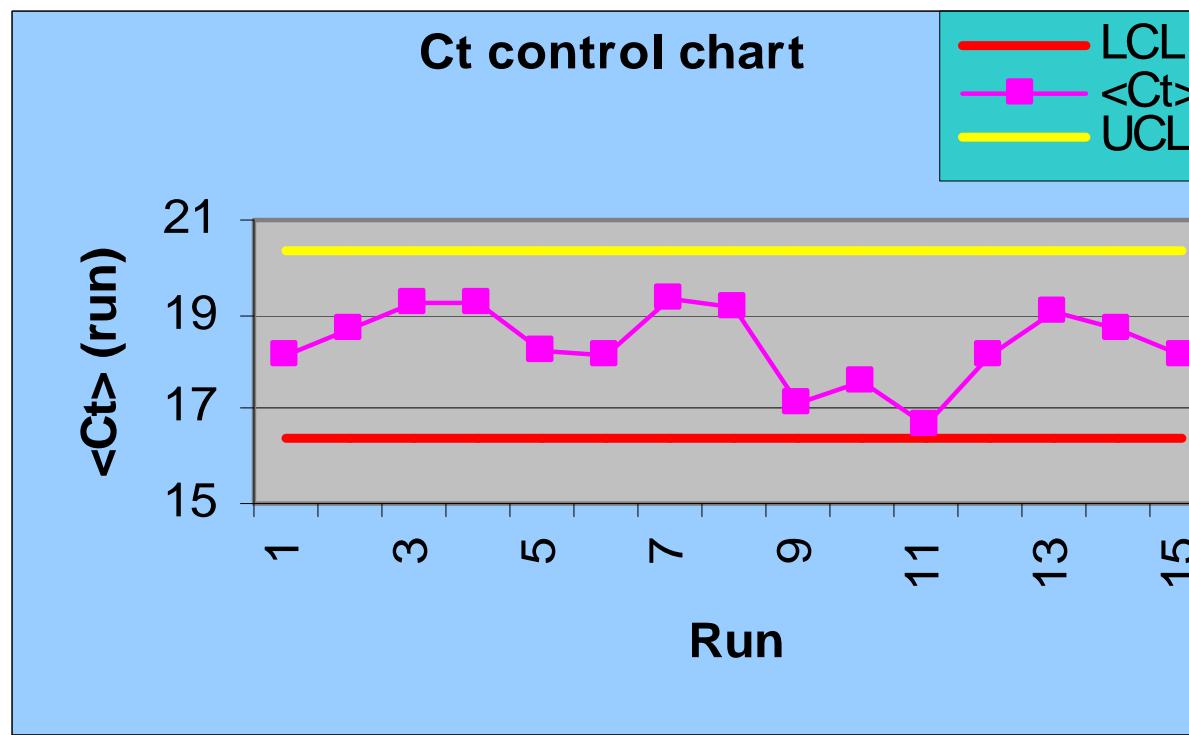
Comparing Initial Target Concentration Acquired on Two Different Platforms



| | <u>7700</u> | | | <u>Mx4000 B01</u> | | | ratio | 1-(p) | Different? |
|--------|-------------|----------|--------|-------------------|----------|--------|-------|--------|------------|
| | <u>Ct 1</u> | <u>n</u> | St Qty | <u>Ct 2</u> | <u>n</u> | St Qty | | | |
| unk1 | 19.78 | 1 | 426.80 | 22.17 | 1 | 476.95 | 1.12 | 54.0% | Not sure |
| unk2 | 23.07 | 1 | 43.16 | 25.73 | 1 | 42.37 | 1.02 | 10.2% | Not sure |
| unk1-2 | 19.61 | 1 | 480.44 | 25.70 | 1 | 43.24 | 11.11 | 100.0% | YES! |

Assay Variability

Intra-Laboratory Variability



LCL and UCL calculated for the Ct-Template is TBP at 100 ng QPCR Reference Total RNA

Summary: Three Main Uses for the QPCR Human Reference Total RNA

- Assay Development:
 - Primer optimization
 - Probe optimization
 - Multiplex compatibility
- Assay Validation:
 - External standard
- “Standardization”:
 - Day-to-day comparison
 - Instrument-to-instrument comparison

Acknowledgements

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